# Pyridoxine responsiveness in novel mutations of the PNPO gene

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Abstract: OBJECTIVE: To determine whether patients with pyridoxine-responsive seizures but normal biomarkers for antiquitin deficiency and normal sequencing of the ALDH7A1 gene may have PNPO mutations. METHODS: We sequenced the PNPO gene in 31 patients who fulfilled the above-mentioned criteria. RESULTS: We were able to identify 11 patients carrying 3 novel mutations of the PNPO gene. In 6 families, a homozygous missense mutation p.Arg225His in exon 7 was identified, while 1 family was compound heterozygous for a novel missense mutation p.Arg141Cys in exon 5 and a deletion c.279$_2$90*delinexon*3.*Pathogenicityoftherespectivemutationswasprovenbyabsencein*100*controlalleles*and*expressionstu*
*K*1*celllines*.*Theresponsetopyridoxinewaspromptin*4, *delayedin*2, *onEEGonlyin*2, *andinitiallyabsentinanother*2*patient*
*phosphate*(*PLP*).*CONCLUSIONS* : *Thisstudychallengestheparadigmofexclusive PLPresponsivenessinpatientswith*
*phosphateoxidasedeficiencyandunderlinestheimportanceofconsecutivetestingofpyridoxineand PLPinneonateswithan*
*resistantseizures*.*PatientswithpyridoxineresponsebutnormalbiomarkersforantiquitindeficiencyshouldundergoPNP*

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# Pyridoxine responsiveness in novel mutations of the *PNPO* gene

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## ABSTRACT

**Objective:** To determine whether patients with pyridoxine-responsive seizures but normal biomarkers for antiquitin deficiency and normal sequencing of the *ALDH7A1* gene may have *PNPO* mutations.

**Methods:** We sequenced the *PNPO* gene in 31 patients who fulfilled the above-mentioned criteria.

**Results:** We were able to identify 11 patients carrying 3 novel mutations of the *PNPO* gene. In 6 families, a homozygous missense mutation p.Arg225His in exon 7 was identified, while 1 family was compound heterozygous for a novel missense mutation p.Arg141Cys in exon 5 and a deletion c.279_290del in exon 3. Pathogenicity of the respective mutations was proven by absence in 100 control alleles and expression studies in CHO-K1 cell lines. The response to pyridoxine was prompt in 4, delayed in 2, on EEG only in 2, and initially absent in another 2 patients. Two unrelated patients homozygous for the p.Arg225His mutation experienced status epilepticus when switched to pyridoxal 5′-phosphate (PLP).

**Conclusions:** This study challenges the paradigm of exclusive PLP responsiveness in patients with pyridoxal 5′-phosphate oxidase deficiency and underlines the importance of consecutive testing of pyridoxine and PLP in neonates with antiepileptic drug–resistant seizures. Patients with pyridoxine response but normal biomarkers for antiquitin deficiency should undergo *PNPO* mutation analysis. ***Neurology*® 2014;82:1425–1433**

## GLOSSARY

**AASA** = α-aminoadipic semialdehyde; **PA** = pipecolic acid; **PLP** = pyridoxal 5′-phosphate; **PNPO** = pyridoxal 5′-phosphate oxidase.

In 2005 and 2006, the molecular background of the 2 most prevalent forms of vitamin $B_6$-dependent epilepsies due to inborn errors of metabolism, namely pyridoxal 5′-phosphate oxidase (PNPO) deficiency and pyridoxine-dependent epilepsy due to antiquitin deficiency, was elucidated.[1,2] Clinically both disorders present with neonatal mixed multifocal myoclonic tonic seizures that may be accompanied by primary poor adaptation, epileptic encephalopathy, and high mortality if causal treatment is delayed. So far, the clinical response to different forms of vitamin $B_6$ has been used to guide further biochemical and molecular workup. Patients with antiquitin deficiency are responsive to pyridoxine and have elevated α-aminoadipic semialdehyde (AASA), while patients with PNPO deficiency are in need of the active vitamer pyridoxal 5′-phosphate (PLP) and lack a specific biomarker.[1,3–5] To date, few mutations of the *PNPO* gene have been further characterized by in vitro expression studies.[1,6,7]

With larger cohorts undergoing biochemical and molecular testing, we observed that a minority of patients with neonatal pyridoxine-responsive seizures did not show the typical biomarker profile of antiquitin deficiency and had wild-type sequences of the *ALDH7A1* gene. This led us to the hypothesis that these patients may have mutations of the *PNPO* gene that allow some residual

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function and that these patients may benefit from higher substrate concentrations. We describe 11 children of 7 families with 3 novel *PNPO* gene mutations with a complete or partial pyridoxine response and expression of mutations in CHO1 cell lines.

**METHODS** **Standard protocol approvals, registrations, and patient consents.** Patient samples were sent from different pediatric centers to the Laboratory of Metabolic Diseases, Department of Pediatrics at the Medical University Hospital Graz for biochemical and genetic workup of pyridoxine-responsive seizures. In all patients, written informed consent of parents had been given for molecular analysis of the *ALDH7A1* gene.

Following diagnostic workup, we identified a total of 34 patients suspected to have pyridoxine-responsive seizures by their referring physician, who had normal biomarkers and wild-type sequence analysis of the *ALDH7A1* gene.

To test our hypothesis, we selected one patient (2b) out of this cohort in whom there was information on a clear pyridoxine response and recurrence of seizures upon a controlled withdrawal. We contacted the referring physician and asked for written informed consent of the parents of patient 2b for molecular analysis of the *PNPO* gene. Having identified sequence anomalies of the *PNPO* gene in this first patient, we subsequently contacted other referring physicians and performed analysis of the *PNPO* gene in another 30 DNA samples as soon as written informed consent for the molecular analysis of the *PNPO* gene of the parents had been obtained.

In those with sequence anomalies of the *PNPO* gene, we asked for detailed information on patient history and results of previous metabolic investigations. No patient underwent a spinal tap for reasons related to this study.

Details on methods regarding biochemical analyses, genotyping, cloning of the *PNPO* gene, site-directed mutagenesis, transient CHO-K1 cell transfection, cell cultures, and enzyme assays are provided as e-Methods (tables e-1 and e-2 on the *Neurology*® Web site at Neurology.org).

**RESULTS** Among the 31 patients analyzed, we were able to identify 3 novel mutations of the *PNPO* gene in 9 living patients from 7 unrelated families. In 2 families, the first child had died due to therapy-resistant seizures. Details on clinical data, biochemical investigations, and mutation analysis are provided in tables 1–3.

**Phenotype, EEG findings, and cranial imaging.** Four out of 11 patients were born prematurely (<full 36 gestational weeks). All patients presented in the neonatal period with recurrent bilateral myoclonic and tonic jerks that were in single patients accompanied by rolling eye movements and desaturation. Neonatal EEG records were available in 9/11 patients and showed burst-suppression patterns in 5 and discontinuous tracings in 4 at week of gestation 37 to 39 and were considered abnormal.

Ten of eleven patients had a first pyridoxine administration within their first week of life, while in one the first pyridoxine administration was delayed to week 6 and in 1 deceased sibling no data were available. Pyridoxine administration led to prompt cessation of seizures in 4 patients, delayed seizure reduction over several days in 2, initial improvement on EEG only in another 2, and had no effect in 2 other patients, one of them receiving a single 100-mg IV dose administration only. One of these 2 initially unresponsive patients underwent a second pyridoxine trial at age 7 months with prompt interruption of seizures. In one patient, no details on the first pyridoxine administration are available. One patient underwent a formal pyridoxine withdrawal at age 3 months with seizure recurrence within 12 hours and accompanying encephalopathy. Two patients developed status epilepticus as soon as pyridoxine was replaced by an identical dose of PLP at the scheduled time of medication administration. Of the 9 alive patients, 6 are on pyridoxine monotherapy, while 3 have comedication with variable antiepileptic drugs. Breakthrough seizures while on pyridoxine were observed in 5 out of 9 patients alive at some point in time. Cranial MRI revealed bilateral encephalomalacia in 1 patient and mild brain atrophy in 2 others. Cranial MRI or ultrasound was normal in 5 and 4 patients, respectively.

Overall outcome was good in 5 unrelated patients at age 1 8/12 years, 1 5/12 years, 6 10/12 years, and 26 years, 2 of them having been index cases. Two patients had global developmental delay and needed special care or support at school. Two patients with markedly delayed continuous administration of pyridoxine had severe neurologic sequelae and 2 affected siblings from 2 unrelated families have died in the absence of continuous pyridoxine treatment.

**Biochemical analyses.** Biochemical testing was performed in samples prior to pyridoxine administration unless outlined otherwise (table 2). AASA or pipecolic acid (PA) as biomarkers for antiquitin deficiency were analyzed in all 9 patients alive and found normal except in patient 4a who had mild elevation of PA in urine at birth and of AASA on a repeat sample at age 2 months. Increased PA concentration in plasma was found in patient 6 and normalized 1 week after the introduction of pyridoxine. Neurotransmitter analysis showed elevated concentrations of amine metabolites in patient 1b in a pretreatment sample, and was normal in patient 2b and patient 6 before and patient 7 while on pyridoxine. Vanillactate in urine was available in only 1 patient, patient 6, and was elevated. Lactate levels in plasma were markedly elevated in the neonatal period in 3 out of 4 patients measured. No patient underwent analysis of PLP concentrations in CSF.

**Genotyping.** There was 100% sequence identity between the patients' *ALDH7A1* gene and the sequence listed for the human antiquitin gene (NM_001182.3).

Sequence analysis of the *PNPO* gene revealed a total of 3 novel mutations in 9 patients from 7 unrelated families as well as 4 polymorphisms (figure, table 3). Within all families, cosegregation of the novel mutation

**Table 1** Clinical data on 11 patients/7 families with pyridoxine-responsive mutations of the *PNPO* gene

| Family/ patient | Birth/neonatal period | Seizure onset | EEG | 1st Administration of pyridoxine | Pyridoxine effect | Current medication | Clinical course and outcome |
|---|---|---|---|---|---|---|---|
| 1a | 33 6/7 GW, Apgar 4/6/8, respiratory distress, ventilation | 1st day (25 min) | BSP | 1st–5th day BDZ + PHB + PHT, 5th day pyridoxine 1 × 100 mg IV SD | None | — | †At age 7 days due to SE |
| 1b | 35 6/7 GW, Apgar 9/8/8, II° CS (pathologic CTG, green amniotic fluid), respiratory distress, ventilation for 3 d, antibiotics for 3 d | 1st day (24 hours) | BSP | 2nd–6th day PHB + PHT, 6th and 9th day pyridoxine 100 mg IV SD, from day 9 10 mg/kg/d | Gradual EEG improvement, seizure-free on pyridoxine + PHB | Pyridoxine 200-150-150-200 mg/d monotherapy since age 9 years | Current age 9 9/12 years, global developmental delay, seizure-free, SE upon switch to identical dose of PLP after 12-h interval |
| 2a | NA | NA | BSP | Yes | No effect | — | †At age 6 months due to refractory seizures |
| 2b | 39 2/7 GW, Apgar 9/10/10, spontaneous delivery, green amniotic fluid | 1st day (3 hours) | NA | 1st day PHB, 2nd day pyridoxine 100 mg IV SD, then pyridoxine 5–7 mg/kg/d | No initial effect, effective with time, encephalopathic upon pyridoxine withdrawal at age 3 months | Pyridoxine 500-0-500 mg/d (33 mg/kg/d), LVT 4 mg/kg/d, DM type 1 | Current age 8 2/12 years, global developmental delay, recurrent single breakthrough seizures during infancy, SE upon switch to PLP 12 hours after last pyridoxine, SON 85 at age 6 y |
| 2c | 35 4/7 GW, Apgar 7/9/9, green amniotic fluid, transient respiratory distress, abdominal distension | 2nd day | BSP | 2nd day pyridoxine 400 mg, then PHB 30 mg IV SD | Interruption of BSP, seizures only stopped + PHB | Pyridoxine 250-0-250 mg/d (30 mg/kg/d) monotherapy since age 3 months | Current age 3 10/12 years, normal development |
| 3 | 39 GW, I° CS, Apgar 8/9/? | 6th day | Discontinuous pattern | 6th day PHB + 50 mg pyridoxine IM SD | Prompt response | Pyridoxine 100 mg/d, SD GBP 1,200 mg/d | Current age 26 years, recurrent SE during infancy and childhood, recurrent febrile seizures until late adolescence, normal IQ, seizure-free for last 4 years |
| 4a | 36 5/7 GW, Apgar 3/6/7, neonatal asphyxia and meconium aspiration syndrome, hypothermia treatment (3 d) under ventilation | Pathologic EEG, followed by clinical seizures | Flat tracing, then BSP | PHB, thiopental, chloralhydrate, at 6 weeks pyridoxine 100 mg IV SD, then 25 mg/kg/d | Prompt response | Pyridoxine 100-0-100 mg/d (20 mg/kg/d), LVT 45 mg/kg/d, PHB 5 mg/kg/d | Current age 4 8/12 years, spastic tetraparesis, progressive microcephaly, severe global developmental delay, recurrent single seizure events |
| 4b | 37 6/7 GW, Apgar 9/9/10, secondary respiratory distress and ventilation (CPAP) | 1st day (11 hours) | Discontinuous pattern | 1st–4th day pyridoxine 100 mg IV, PHB, restarted on day 6 with 30 mg/kg/d | Prompt response | Pyridoxine 100-0-100 mg/d (13 mg/kg/d) monotherapy since age 9 months | Current age 1 8/12 years, unbalanced gait, otherwise normal development, seizure-free |
| 5 | 38 1/7 GW, Apgar 9/10/9, postnatal respiratory distress and asphyxia, hypothermia (4 d) and antibiotic (6 d) treatment | 6th day (eventually 1st day) | Discontinuous pattern | 6th day pyridoxine 30 mg/kg/d PO + PHB 7 mg/kg/d | Seizure-free within 3 days | Pyridoxine 100-0-150 mg/d (21 mg/kg/d) monotherapy since age 4 months | Current age 1 5/12 years, normal development, unsteady gait, seizure-free |
| 6 | 34 GW, abdominal distension | 2nd day | NA | Received pyridoxine in the neonatal period at age 7 months, pyridoxine, 100 mg IV | No response, prompt response | Pyridoxine 300-200-300 mg/d (50 mg/kg/d) | Current age 8 years, spastic tetraparesis, secondary microcephaly, cognitive deficit, seizure-free, relapses upon febrile infections |
| 7 | 38 2/7 GW, Apgar 9/10/10 | 1st day (3 hours) | Discontinuous pattern | 1st day | Prompt response to recurrent IV administration | Pyridoxine 100-100-200 mg/d (17 mg/kg/d), OXC 13 mg/kg/d | Current age 6 10/12 years, development normal, seizure-free over last 7 months |

Abbreviations: BDZ = benzodiazepines; BSP = burst-suppression pattern; CPAP = continuous positive airway pressure; CS = cesarean section; CTG = cardiotocogram; DM = diabetes mellitus; GBP = gabapentin; GW = gestational week; IM = intramuscular; LVT = levetiracetam; NA = not available; OXC = oxcarbazepine; PHB = phenobarbitone; PHT = phenytoin; PLP = pyridoxal 5′-phosphate; SD = single dose; SE = status epilepticus; SON = Snijders-Omen nonverbal intelligence test.
† Deceased.

**Table 2** Biochemical profiles of 11 patients/7 families with pyridoxine-responsive mutations of the *PNPO* gene

| | Patient 1a | Patient 1b | Patient 2a | Patient 2b | Patient 2c | Patient 3 | Patient 4a | Patient 4b | Patient 5 | Patient 6 | Patient 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **AASA** | | | | | | | | | | | |
| Urine (ref <2 [<0.5 mo], <1 [0.5–1 y], <0.5 [>1 y] mmol/mol Crea) | NA | NA | NA | NA | NA | <1[a] | <1 at 1 mo, 1.3 at 2 mo | <1 | <1 | <1 | NA |
| CSF (ref < 0.1 μmol/L) | NA | Normal | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| **Pipecolic acid** | | | | | | | | | | | |
| Plasma (ref 3.75–10.8 [<1 wk], 0.7–2.46 [>1 wk] μmol/L) | NA | Normal | NA | Normal[a] | Normal | Normal[a] | NA | NA | NA | 20 at 7 mo | Normal |
| Urine | NA | NA | NA | Normal[a] | Normal | NA | Elevated | Normal | Normal | NA | Normal |
| CSF (ref 0.009–0.12 μmol/L) | NA | Normal | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| **Lactate** | | | | | | | | | | | |
| Plasma (ref <2 mmol/L) | NA | NA | NA | 2.2 | NA | NA | 12.4 | 11 | 14.9 | NA | NA |
| **Amino acids (plasma)** | | | | | | | | | | | |
| Threonine, μmol/L | NA | 82 on day 3 (ref 22.2–52.6) | NA | Normal | NA | Normal | Normal | Normal | Normal | Normal | Normal |
| Glycine, μmol/L | NA | Normal | NA | Normal | NA | Normal | 1,102 at birth (ref 232–740) | Normal | Normal | Normal | Normal |
| Arginine, μmol/L | NA | Normal | NA | 142 at 9 wk (ref 6–130) | NA | Normal | Normal | Normal | Normal | Normal | Normal |
| **Neurotransmitters** | | | | | | | | | | | |
| HVA | NA | Normal | NA | Normal | NA | NA | NA | NA | NA | Normal | Normal[a] |
| 5-HIAA, nmol/L | NA | 891 on day 3 (ref 150–800) | NA | Normal | NA | NA | NA | NA | NA | Normal | Normal[a] |
| 3-0-methyl dopa, nmol/L | NA | 3,879 on day 3 (ref <300) | NA | Normal | NA | NA | NA | NA | NA | Normal | Normal[a] |
| **Organic acids** | | | | | | | | | | | |
| Urine | NA | Elevated lactate on day 2 | NA | Normal | NA | NA | Elevated lactate on day 2 | Elevated lactate on day 1 | Normal | NA | Normal |
| **Vanillactate** | | | | | | | | | | | |
| Urine | NA | NA | NA | NA | NA | NA | NA | NA | NA | Elevated at 7 mo | NA |

Abbreviations: AASA = α-aminoadipic semialdehyde; HVA = homovanillic acid; HIAA = hydroxindoleacetic acid; NA = not available; ref = reference.
[a] On pyridoxine.

Table 3 Mutation analysis of the *PNPO* gene in 11 patients from 7 unrelated families: Reference sequence NM_018129.3

| Patient | Mutation | Exon | Polymorphisms | Country of origin |
|---|---|---|---|---|
| 1a | NA | | NA | Kosovo |
| 1b | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.347G>A] homozygote | |
| | | | [IVS6-26A>G] homozygote | |
| | | | [c.552G>A] homozygote | |
| 2a | NA | | NA | Albania |
| 2b | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.347G>A] homozygote | |
| | | | [c.552G>A] homozygote | |
| 2c | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.347G>A] homozygote | |
| 3 | [c.421C>T] + [c.279_290del]; (p.Arg141Cys + p.Ser93Ser, Ala94_Leu97del) | 5/3 | [c.347G>A] [IVS6-26A>G] | Canada |
| | | | [c.552G>A] homozygote | |
| | | | [c.165C>T] | |
| 4a | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.552G>A] homozygote | Kosovo |
| 4b | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.552G>A] homozygote | |
| 5 | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | [c.552G>A] homozygote | Serbia |
| 6 | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | | Unknown |
| 7 | [c.674G>A] + [c.674G>A]; (p.Arg225His + p.Arg225His) | 7 | c.[347G>A] homozygote | Former Yugoslavia (no details) |
| | | | [c.552G>A] homozygote | |

Abbreviation: NA = not available.

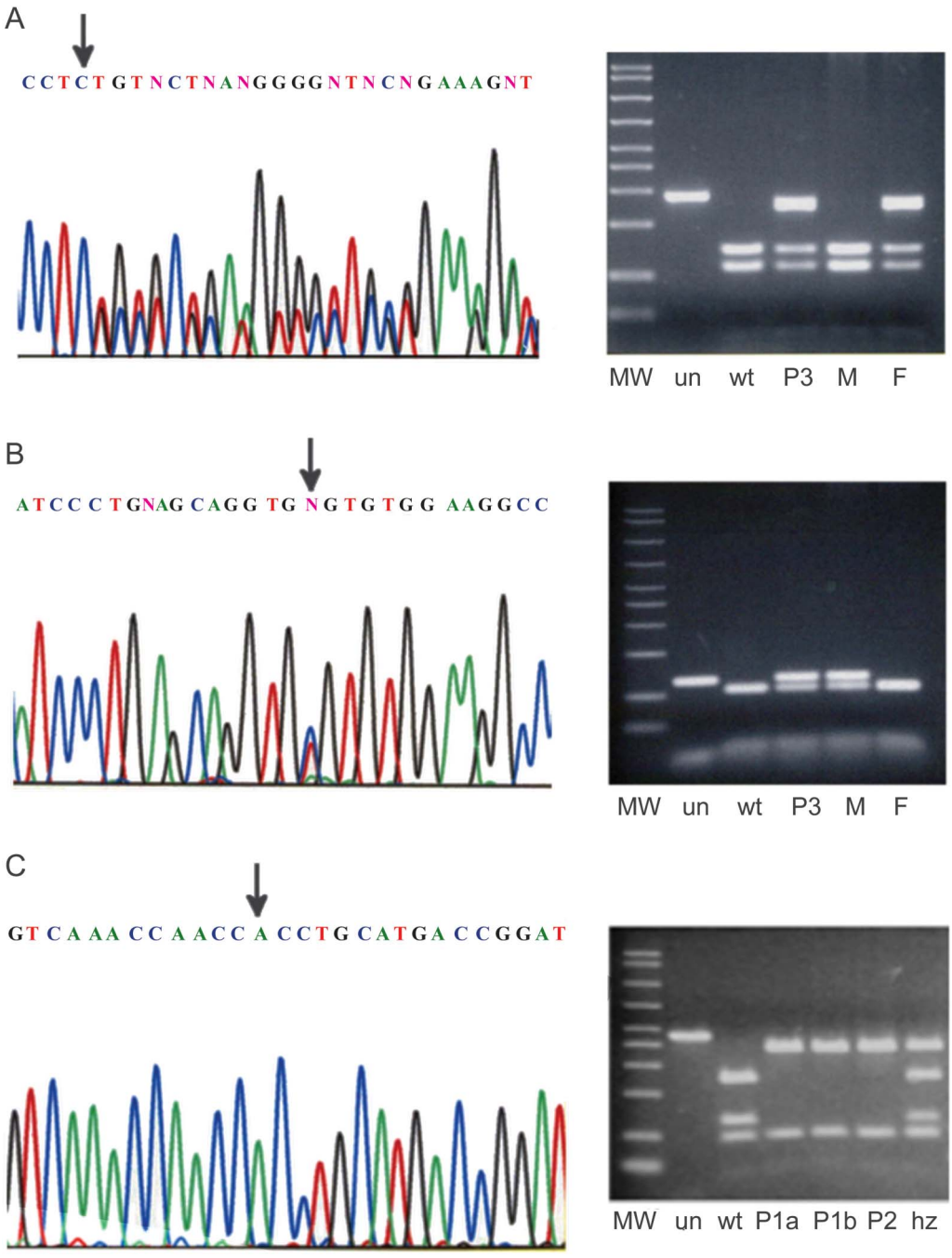
was proven by heterozygous state in both parents and in 2 unaffected siblings of family 1. No material of the deceased siblings 1a and 2a and of the unaffected sibling of patient 2a and patient 3 was available for molecular testing.

The novel missense mutations, p.Arg225His as well as p.Arg141Cys, affect arginine residues presenting codons with the potential CpG DNA methylation site with an increased probability for mutational events. The novel deletion c.279_290del is located in the β-strand S2 of the PNPO protein. None of the novel mutations could be found in 100 control alleles. In addition, a total of 4 polymorphisms were identified (table 3). Among the 4 polymorphisms, c.347G>A had a high prevalence of 2.7% on 200 alleles tested.

**Expression studies in CHO-K1 cell lines (Laboratory of Metabolic Diseases Graz).** In vitro studies in CHO-K1 cell lines showed that transfection with wild-type PNPO produced readily measurable enzyme activity. Transfection with DNA containing the p.Arg225His mutation resulted in undetectable enzyme activity (n = 10). Transfection with DNA containing the p.Arg141Cys resulted in reduced enzyme activity of ~51% of that of wild-type (n = 10). Transfection with DNA containing the c.279_290del resulted in undetectable enzyme activity (n = 10).

**DISCUSSION** With respect to their clinical presentation, our 11 patients with pyridoxine-responsive *PNPO* mutations were indistinguishable from previously reported cases with exclusive PLP response. In the 24 patients with PNPO deficiency harboring a total of 10 different mutations published to date,[1,3–6,8–10] only 2 have shown a partial pyridoxine response with improvement when switched to PLP.[1,8] In contrast, all 9 patients harboring specific novel *PNPO* mutations published in this article had partial or even complete pyridoxine response and were thus initially suspected to have antiquitin deficiency by their referring physician. Antiquitin deficiency was excluded by normal AASA or PA concentrations in all families and by wild-type sequence of the *ALDH7A1* gene in every living patient. In antiquitin deficiency, 85% of a larger patient series had prompt response to the first administration of pyridoxine.[11] In this series, only 4 out of 9 patients (44%) had a prompt initial pyridoxine response, while 2 had delayed effects with reduction of seizures over days, 2 by effect on EEG only, and 2 had failure of initial response in the neonatal period. Patient 2b underwent a formal pyridoxine withdrawal with rapid recurrence of seizures 12 hours after last intake, while in 2 cohorts of patients with confirmed antiquitin deficiency interval to seizure recurrence ranged from 24 hours to weeks.[12–14] Complete seizure freedom or occasional seizures on PLP monotherapy have so far been reported in single patients with PNPO deficiency.[10,14,15] Of interest, in our cohort of patients with pyridoxine-responsive *PNPO* mutations, 6 patients (66%) were completely seizure-free on pyridoxine

**Figure** Mutations found in the human *PNPO* gene




(A) Left column: deletion c.279_290del (p.Ser93Ser, Ala94_Leu97del) in exon 3. Right column: restriction digest test: PCR with loss of restriction site (restriction site alteration—Cac8I). Patient 3 and his father are heterozygous. (B) Left column: missense mutation c.421C>T (p.Arg141Cys) in exon 5. Right column: restriction digest test: PCR with loss of restriction site upon introduction of a mismatch primer (restriction site alteration—*Mvn*I*). Patient 3 and his mother are heterozygous. (C) Left column: missense mutation c.674G>A (p.Arg225His) in exon 7. Right column: restriction digest test: PCR with introduction of a restriction site (restriction site alteration + *Bsp*MI). Patients 1b, 2b, and 2c (4a, 4b, 5, 6, and 7 not shown) were homozygous. Heterozygote (Hz) state as identified in parents of families 1, 2, 4, 5, 6, and 7. F = father; M = mother; MW = molecular weight; P = patient; un = undigested PCR; wt = wild-type.

monotherapy, in some with only 2 single doses a day. Still, these pyridoxine-responsive *PNPO* mutations cannot be regarded as "mild," as 2 siblings have died from epileptic encephalopathy in the absence of continuous pyridoxine treatment and 2 patients with treatment delay are severely handicapped. No patient in this study received PLP prior to the identification of the *PNPO* mutation. As a result of our genetic findings, 2 patients, who by then still had occasional seizures, were switched to PLP. Both patients developed status epilepticus as soon as pyridoxine was replaced by an identical dose of PLP at the scheduled

time of medication administration. Thus all patients continued on pyridoxine with or without comedication of other antiepileptic drugs (table 1).

This phenotype of pyridoxine responsiveness was exclusively associated with 3 novel mutations of the *PNPO* gene, namely a deletion c.279_290del in exon 3, a missense mutation p.Arg141Cys in exon 5, and another missense mutation p.Arg225His mutation in exon 7 of the *PNPO* gene. In order to interpret the damaging effect of these novel mutations, we applied the computational software tools SIFT[16] and PolyPhen-2,[17] which analyze the impact of sequence variations on protein function. In addition, pathogenicity of the novel mutations was assessed by expression studies in CHO-K1 cells for all 3 mutations.

The novel mutation p.Arg225His in exon 7 was present on 16/18 alleles and is affecting the PLP binding site in a strongly conserved region of the PNPO protein (table e-2).[18] Prediction results by PolyPhen-2 about the probability of damage revealed a maximal score of 1.0 by HumDiv and 0.999 by HumVar. Prediction results by SIFT gave a maximal probability for disturbed protein function of 0.0. Multiple sequence alignment showed 100% conservation among 60 different species by PolyPhen-2 and a high median sequence conservation of 3.02 among 33 different species by SIFT.

The novel mutation p.Arg141Cys in exon 5 was found in 1 compound heterozygous patient and is part of the highly conserved region carrying the FMN binding site, which by forming 2 hydrogen bonds between the molecule and the enzyme plays an important role in the enzymatic reaction.[18] Prediction results by PolyPhen-2 revealed a maximal score of 1.0 by HumDiv and 0.998 by HumVar. Prediction results by SIFT gave a maximal probability for disturbed protein function of 0.0. Multiple sequence alignment showed conservation in 54 out of 55 different species by PolyPhen-2 and a high median sequence conservation of 3.03 by SIFT.

The novel deletion c.279_290del in exon 3 was found in compound heterozygosity in 1 patient and leads to the loss of a highly conserved residue, arginine 95, most probably inhibiting the formation of the β-strand. None of the lost amino acids is involved in pyridoxine 5′-phosphate/pyridoxamine 5′-phosphate oxidation.[18] Although there is no frameshift in the protein sequence, one can speculate that the protein structure is not stable and no functional enzyme is translated.

Expression studies in transfected CHO-K1 cell lines revealed a 50% reduced PNPO enzymatic activity for mutation p.Arg141Cys, while transfection with p.Arg225His and c.279_290del led to no detectable enzymatic activity in our in vitro expression system. No Western blot studies have been performed to show if mutant proteins were expressed and processed. Still, these results underline the pathogenicity of each respective mutation but may be an underestimate of in vivo residual enzyme activity due to potential stabilization of mutant PNPO protein in its natural environment. The observed pyridoxine response suggests some residual enzymatic activity for p.Arg225His found in homozygous form in 8 patients. Alternatively, pyridoxine could have a chaperon effect and preserve the mutant PNPO protein from premature decay.

As 6 out of all families carrying the p.Arg225His mutations derive from former Yugoslavia, it may well represent a founder mutation. The main function of PNPO is PLP formation,[18] which serves as an essential cofactor in over 120 enzyme reactions in amino acid and neurotransmitter metabolism. More recently, a role of PNPO in intracellular recycling of cofactor degraded enzymes[18] and channeling of PLP to its various apoenzymes has been described.[19] In order to protect cells from PLP toxicity, PLP has a strong inhibitory effect on PNPO activity.[20] The observed status epilepticus in 2 patients homozygous for the novel p.Arg225His mutation suggests that the PNPO protein carrying the mutation p.Arg225His might be completely inhibited by the administration of PLP. Further in vitro studies are needed to prove this hypothesis.

While antiquitin deficiency is biochemically characterized by a specific compound, AASA, or less specifically by elevated PA in urine, plasma, and CSF,[2,21,22] PNPO deficiency lacks a specific biomarker. Low PLP concentrations in CSF have previously been considered a hallmark of PNPO deficiency,[23] but can be found in several inborn errors affecting PLP metabolism,[24] are thus unspecific, and require sampling prior to PLP or pyridoxine administration. Secondary deficiency of homovanillic acid hydroxyindoleacetic acid and increased concentrations of 3-methoxytyrosine have been described in several PNPO patients[1,5] but are only present in about half of all PNPO patients.[25] Still, these secondary findings can be helpful in raising the suspicion of a disorder related to cerebral vitamin $B_6$ deficiency. Of interest, one patient from our series had elevated concentrations of neurotransmitter amine metabolites. This seems paradoxical but has so far been reported in 3 other patients with PNPO deficiency.[4,6,15] Elevation of urinary vanillactate due to the buildup of L-dopa is present in about 80% of PNPO patients and has been raised in the one patient from this series who underwent determination. Recently, distinct $B_6$ vitamer profiles have been described in the plasma of patients with PNPO deficiency, even when on treatment.[26] No measurement of PLP in CSF or investigation of $B_6$ vitamer profiles in plasma have been performed in any patient presented in this study.

The findings of our study have several important implications for our clinical practice. First, they shift the paradigm of exclusive PLP responsiveness of patients with PNPO deficiency and contradict the strategy of using PLP instead of pyridoxine as the first-line

vitamin to test for all inborn errors with vitamin $B_6$-responsive seizures. Second, they underline the importance of testing for *PNPO* mutations in the presence of a pyridoxine response but normal biomarkers, especially those of antiquitin deficiency or other vitamin $B_6$-dependent epilepsies that can manifest in the neonatal period such as severe congenital hypophosphatasia.[27–29] Considering the high preponderance of birth asphyxia in antiquitin as well as PNPO deficiency, a standardized trial with pyridoxine and PLP should be performed routinely in neonates with seizures irrespective of the birth history. Third, this study suggests that patients with pyridoxine-responsive mutations in the *PNPO* gene might be a prevalent group, considering a total of 24 patients with PLP-responsive *PNPO* mutations published to date and 11 new patients with pyridoxine-responsive mutations of the *PNPO* gene. The challenge of recognizing a delayed pyridoxine effect and current lack of specific biomarkers puts this patient group at a considerable risk of remaining undiagnosed.

## AUTHOR CONTRIBUTIONS

B.P. is first author and guarantor of this manuscript, raised the hypothesis, made the concept, interpreted all data, and drafted this manuscript. K.P. performed the molecular analysis of the *PNPO* and *ALDH7A1* gene and interpreted the data. P.M. performed molecular analysis of patients 1b and 7 and gave important intellectual input to this manuscript. P.C. interpreted molecular data and gave important intellectual input to this manuscript. E.P. interpreted molecular data and expression studies in CHO1 cells. O.M. characterized family 1 and gave important intellectual input to this manuscript. O.H. characterized patient 7 and gave important intellectual input to this manuscript. G.S. characterized family 4 and 5 and gave important intellectual input to this manuscript. S.K. characterized family 2 and gave important intellectual input to this manuscript. M.C. characterized patient 3 and gave important intellectual input to this manuscript. N.W. characterized patient 6 and gave important intellectual input to this manuscript. E.S. measured the α-aminoadipic semialdehyde and gave important intellectual input to this manuscript. S.S. gave important intellectual input to this manuscript. L.A. was active in data acquisition and interpretation and in drafting the tables, figures, and text elements. All authors have approved the final version of this article.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